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Stanislawski L, Lefeuvre M, Bourd K, Soheili-Majd E, Goldberg M, Perianin A (2003). TEGDMA-induced toxicity in human fibroblasts is associated with early and drastic glutathione depletion with subsequent production of oxygen reactive species. J. Biomed. Res. 66:476-82.

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Full Length Research paper

Detection of cytomegalovirus (CMV) antibodies or DNA sequences from ostensibly healthy Iranian mothers and their neonates

Seyed Hamidreza Monavari¹*, Hossein Keyvani², Bahman Abedi Kiasari³, Hamidreza Mollaei², Mehdi Fazlalipour², Mostafa Salehi Vaziri² and Fatemeh O. Fallah⁴

¹Department of Virology and Antimicrobial Resistance Research Center, Tehran University of Medical Sciences, Tehran, Iran.

²Department of Virology, Tehran University of Medical Sciences, Tehran, Iran.

³Human Viral Vaccine Department, Razi Vaccine and Serum Research Institute, Hesarak, Karaj, Iran.

⁴Paediatric Disease Department, Valiasr Hospital, Imam Khomeini Hospital, Tehran University of Medical Sciences, Tehran, Iran.

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Cytomegalovirus (CMV) remains the most common cause of viral intrauterine infection. The objective of this research was to determine the prevalence of at-risk pregnancies for congenital cytomegalovirus transmission in a randomly selected pregnant women and their newborns. Enzyme Link Immunosorbent Assay (ELISA) and real-time polymerase chain reaction (PCR) were utilized to screen the sera of mothers (n = 100) and consecutive umbilical cord blood samples from their newborn (n = 100) 100). Of the 100 mother's sera analyzed, 100 (100%) and 3 (3%) were positive for cytomegalovirus IgG and IgM antibodies, respectively. Of the 100 cord serum specimens analyzed, 99 (99%) and 2 (2%) were positive for cytomegalovirus IgG and IgM antibodies, respectively. Cytomegalovirus DNA was detected in 4 out of 100 (4%) cord blood samples of newborns. From four CMV DNA positive cases, Case 1 had no IgM in cord serum, but had IgM in mother's sera. Cases 2 and 4 were positive for IgM in both mother's sera and cord serum. Case 3 had no detectable CMV IgM in sera and cord serum. As many as 66 and 100% of CMV IgM-positive women in this study also had CMV IgM and CMV DNA in their delivery cord blood samples, respectively suggesting an increased risk of congenital CMV infection in those pregnancies. A paired women sera/cord blood CMV IgM-negative was found to be positive for CMV DNA. The data may also suggest the utility of PCR in place of CMV IgM as a diagnostic method for congenital CMV infection.

Key words: Cytomegalovirus, cord blood, congenital disease, real-time polymerase chain reaction (PCR), antibodies.

INTRODUCTION

Human cytomegalovirus (HCMV) remains the most common cause of viral intrauterine infection and the major cause of congenital defects in developed countries for over 20 years (Munro et al., 2005; Boppana et al., 2001). Involvement of CNS is the most common and important clinical manifestations of congenitally infected infants (Schleiss and Choo, 2006; Dollard et al., 2007).

Transmission can occur during pregnancy or after birth, from breast milk, cord blood, saliva, urine, fomites and other sources (Lazzarotto et al., 2004). Some of infants are infected during delivery, through breast milk or following the transfusion of CMV-contaminated cord blood to a sero-negative newborn that is not protected by maternal antibodies. Congenital CMV infection is identified by viral isolation from the infant at birth or within

^{*}Corresponding author. E-mail: hrmonavari@yahoo.com. Tel/Fax: +98(021)88602205.

3 weeks of birth and diagnosis beyond that age may indicate acquired infection from exposure to virus in the birth canal or breast milk (Daiminger et al., 2005; Revello and Gerna, 2002). Cord blood is a sample of blood collected from the umbilical cord at the time of birth and may help mother to child transmission of CMV and causes congenital CMV infection. Classically, the gold standard diagnostic test for congenital CMV infection in cord blood has been viral isolation but the cell culture of CMV takes long time and expensive. Alternative approach, polymerase chain reaction (PCR) is especially promising for its high sensitivity, specificity and relative simplicity (Demler et al., 1998; Xu et al., 1993).

The presence of CMV DNA in cord blood of infected pregnant women could be a risk marker for transmission of the virus to the fetus. Screening for congenital CMV may lead to more accurate diagnosis of infant mortality and newborn screening may help identify both asymptomatic and misdiagnosed cases of congenital CMV. The study determined the prevalence of at-risk pregnancies for congenital cytomegalovirus transmission in a randomly selected population by detection of cytomegalovirus specific antibodies (IgG and IgM) in the sera of the mothers and cytomegalovirus specific antibodies (IgG and IgM) and DNA in cord bloods from their newborns.

MATERIALS AND METHODS

Specimen collection and processing

Two groups of specimens were included in the study: Group I consisted of serum specimens taken from 100 pregnant women who visited the Clinic of Rasoul Akram Hospital, Tehran, Iran, immediately after delivery in the period of April, 2010 and May, 2011 were included in the study. Group II consisted of consecutive cord blood specimens (n = 100) taken from their newborn cord in the period of April, 2010 and May, 2011. The method of collection and processing of cord blood has been described (Kogler et al., 1996). Patients gave their full informed consent at the time the samples were taken. Tehran University of Medical Science (TUMS) ethics committee approval was granted in March, 2010 to test these samples for the presence of congenital infections. The specimens were stored in the cord blood and serum bank of the Tehran University of Medical Science (TUMS) separately.

Real-time polymerase chain reaction (PCR) assay

Nucleic acid extractions were performed manually with a High pure viral nucleic acid kit (Roche, Germany), according to manufacturer's instructions and stored at -20 °C until use. The real-time PCR was carried out using SYBR Green Master Mix reagents containing 5 units Taq polymerase, 0.01% gelatin, 0.6 μ M of each primer, 200 μ M of each deoxynucleotid triphosphate, 5 μ I of reaction buffer (50 mM KC1, 10 mM tris-HCI, pH = 8.3) and 1.5 mM MgCl₂. Samples were subjected to 1 cycle at 50 °C for 2 min and at 95 °C for 10 min, followed by 40 cycles at 95 °C for 20 s, 60 °C for 40 s using a thermal cycler (Rotor Gene 6000, Corbett Research). SYBR Green I fluorescence was detected and plotted using Rotor-gene 6000 series software (Corbett Research) during the 60 °C extension phase for each cycle. Threshold cycles and log starting quantities

for patient samples were calculated. Forward and reverse primers were as follows: GTGTGGGACATAGGCCAGAG and GCGACATCC CCGCCTACTAC (201-bp product). Standard precautions were taken to avoid sample-to-sample contamination and PCR product carry-over. Sterile water, normal serum control and a negative serum (non reactive on multiple assays) were used as negative controls. CMV plasmid containing gpB region was used as positive control.

Serological assay

Paired sera and cord serum samples collected from 100 pregnant women and their newborn were examined for the presence of CMV IgG and IgM. Enzyme link immunosorbent assay (ELISA) was carried out using commercial ELISA kit (Third generation ELISA kit; RADIM, Italy).

RESULTS

Patient characteristics

The median age of the 100 pregnant women was 34 years. The age distribution of the study population was: < 29 years with one birth; 29.1%; > 29 years with more than one birth; 70.9%. The male to female ratio of babies was 1.31:1. There were no signs of primary infections during pregnancy in pregnant women. Their pregnancies were uncomplicated. All babies were delivered at term and were apparently healthy. They had a normal birth weight, height and head circumference. Thirty percent of these infants were born by cesarean section. All mothers and their children were discharged from the hospital 4 days after delivery. Clinical data of CMV positive patients are shown in Table 1.

Cytomegalovirus polymerase chain reaction (PCR) and antibody assays

The results of the cytomegalovirus PCR and antibodies tests in women sera and cord blood are presented in Table 2. Consecutive umbilical cord blood samples from newborn (n = 100) were analysed for cytomegalovirus DNA using real-time PCR. Cytomegalovirus DNA was detected in 4 out of 100 (4%) cord blood samples from newborns. Paired women sera and cord serum samples (n = 100) were analysed for IgG antibody levels against CMV using ELISA. Of the 100 mother's serum samples and 100 cord serum samples analysed, 100 (100%) and 99 (99%) have detectable levels of CMV IgG antibodies respectively. Sera samples of pregnant mothers (n = 100)and cord serum (n = 100) were analysed for IgM antibody levels against CMV using ELISA. Of the 100 sera and 100 cord serum samples analysed, 3 (3%) and 2 (2%) have detectable levels of CMV IgM antibodies respectively. In total, 4 cases had CMV IgM or CMV DNA identified in women sera or cord blood specimens of their neonates. Case 1 had no IgM in cord serum, but had IgM

	Mothers					Infants					
Pair no.	Age	Gestational age (week)	Mode of pregnancy	Mode of delivery	Prior pregnancy	Sign of infection	Sex	Birth weight (g)	Height (cm)	Head circumference	Sign of infection
1	33	38	Uncomplicated	Spontaneous	1	None	Male	2900	50	33	None
2	35	38	Uncomplicated	Spontaneous	1	None	Female	3200	55	35	None
3	27	38	Uncomplicated	Caesarean	0	None	Female	3000	49	35	None
4	29	40	Uncomplicated	Spontaneous	0	None	Male	3300	52	36	None

Table 1. Clinical presentation and demographics of CMV positive cases.

Table 2. Comparison of the ELISA and PCR results for CMV positive patients.

	Mothers		Infants				
Pair no.	Sera IgG	Sera IgM	Cord serum IgG	Cord blood IgM	Cord blood PCR		
1	+	+	+	-	+		
2	+	+	+	+	+		
3	+	-	+	-	+		
4	+	+	+	+	+		

in sera and CMV DNA in cord blood. Cases 2 and 4 were identified as positive for IgM in both sera and cord serum and CMV DNA in cord blood. Case 3 had no detectable CMV IgM in sera and cord serum; however she had CMV DNA in cord blood.

DISCUSSION

The seroprevalence of CMV varies according to studies conducted in different parts of the world. But, it has been reported that 0.2 to 2% of live birth have congenital CMV infection, considering as the leading cause of congenital infections worldwide (Demmler, 1991; Brown and Abernathy, 1998; Kumar et al., 1973). The fetus is at risk of acquiring CMV infection either intrauterine or delivery. Intrauterine transmission of CMV infection may occur following either primary or recurrent infection (Ahlfors et al., 1988; Fowler et al., 1992). Involvement of central nervous system (CNS), including late central nervous system sequelate, primarily sensoryneuronal deafness is the most important clinical manifestation in 10 to 20% of such CMV congenital infected infants. Screening of mothers for CMV and early diagnosis play an important role to minimize CMV congenital infection and its serious consequences.

Molecular and serological assays were utilized to screen CMV congenital infection in pregnant women sera and cord blood of their newborns at the time of delivery and analyzed the distribution of CMV infection in a group of population with no history of CMV infection. The study showed a frequency of 4% for CMV infection determined by PCR in cord blood samples from newborns compared with 2% CMV infection identified by IgM assay and 99% IgG positivity; 100% IgG positivity and 3% IgM positivity in their mothers as tested by ELISA.

The seroprevalence of CMV IgG antibody in pregnant women (100%) and the frequency of maternal infection (3%) obtained in this study is similar to the previous study in Iran (Tabatabaee and Tayyebi, 2009). They studied the prevalence of CMV antibody in 1472 pregnant women in the period of January, 2007 and July, 2007. The study reported a frequency of 97.69% for the seroprevalence of CMV IgG antibody and an active maternal CMV infection in 4.3% of the population. In our neighbour country, Turkey, the rate of CMV seropositivity was reported to be

98.5% and the prevalence of maternal CMV infection was reported to be 1.2% (Satilmis et al., 2007). A study in Cuba (Kourí et al., 2010) screened 1131 pregnant women for the presence of CMV IgG and IgM in the period of 2007 to 2008. They found that most women (92.7%) were CMV IgG seropositive. Primary and active non-primary infection was reported in 20 (1.77%) and 7(0.62%) of pregnant women.

It was reported that the rate of CMV transmission in seropositive mothers undergoing recurrent infection and pregnant women with primary infection is 0.2 to 2% and 20 to 40%, respectively (Boppana et al., 1999; Stagno and Whitley, 1985). As the seroconversion of pregnant women studied here was not previously monitored, the detection of CMV IgG does not lead to suspicion of primary infection. The study did not perform IgG avidity assay and the differentiation of primary and non primary infection is difficult. IgM assay was performed and 3% of pregnant women and 2% of cord serum of their newborns showed IgM positivity. Only two cord serum samples have detectable CMV IgM antibodies that is less than the IgM positivity in their corresponding mothers (n = 3). With the fact that IgM does not cross the placental barrier, these two babies may have intrauterine CMV infection. Detection of specific CMV IgM antibodies is a serological based method for diagnosis of primary CMV infection (Mace et al., 2004). However, different situations can be associated with the presence of IgM antibodies including the recurrent infection, the convalescent phase of a primary infection, the persistence of IgM or IgM crossreactivity due to herpes viruses other than CMV (Hodinka, 2003). Detection of IgM may be related to a primary infection occurring during pregnancy, although IgM can also be detected during reactivations. It has been previously proved that the main risk of intrauterine infection is associated with a primary infection during pregnancy (Fowler et al., 1992). However, with the fact that none of pregnant women in this study showed clear history of CMV infection during pregnancy and no IgMpositive pregnant mother or positive baby gave symptoms and signs suggestive of CMV infections suggesting that intrauterine infection had occurred from a reactivated, asymptomatic infection with CMV.

The study revealed a variation in ELISA and PCR results between mother sera and cord blood. Due to placental transfer of IgG, all mother's serum and cord serum samples revealed identical results for IgG antibodies against CMV. Paired mother sera/cord serum in 2 cases (case 2 and 4) showed IgM positivity in both sera and cord serum and DNA positivity in cord blood suggesting an active CMV infection in both mother and her baby. As the IgM does not cross the placental barrier, the IgM obtained in cord serum samples could originated from an active CMV infection of CMV DNA in cord blood by PCR may support this hypothesis. Case 3 had no detectable CMV IgM in sera and cord serum; however

she had CMV DNA in cord blood suggesting an active CMV infection diagnosed only by PCR. Case 1 had no IgM in cord serum, but had IgM in sera and CMV DNA in cord blood suggesting an active CMV infection in both mother and baby. The most interesting finding in this study was that in ceases 1 and 3, PCR could detect CMV DNA in cord blood samples, while there was no detectable IgM in sera of mother (case 3) and cord serum (cases 1 and 3). Although, IgM assay is still considered as a reasonable tool for congenital CMV infection diagnosis (Melish and Hanshaw, 1973) it was reported that only 45 to 80% of babies congenitally infected with CMV could be recognized by detection of IgM (Griffiths et al., 1982). These data are further supported by the present study with the finding that in two cases of the 4 congenital infected cases identified by PCR could IgM be found. The data show that the rate of congenital CMV infection detected by PCR (4%) is higher than that detected by IgM assay (2%). The utility of PCR in place of CMV IgM as a diagnostic method for congenital CMV infection is the most important finding of this study. PCR has been previously recognized as an important screening tool for congenital CMV infection. Also, to differentiate an active IgM case from non-active or false positive IgM case, PCR and virus detection has been suggested (Demler et al., 1998; Kourí et al., 2010). CMV DNA detected in specimens from 9 out of the 27 pregnant women by PCR. They suggested that mothers with active infection are at risk to have congenitally infected children. Also, no specific IgM could be in the fetal sera, the methods serological are unable to show this transmission. Thus, PCR method used here is the most suitable technique to screen CMV infection in newborns.

Rapid screening and accurate diagnosis of active cytomegalovirus (CMV) infection are needed for complication prediction, treatment, infection control, and reducing the severity of the disease. PCR and an antibody assay are common methods for cytomegalovirus (CMV) infection detection, however, PCR may lead to more accurate diagnosis of maternal and congenital CMV infection. Future studies with larger number of samples and follow up the positive cases are required to determine the clinical impact of congenital CMV infections.

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Full Length Research paper

A retrospective study of clinical Streptococcus pneumoniae isolates from four health facilities in **South-West Nigeria**

Iwalokun Bamidele A.^{1,3}*, Fowora, M.², Akinloye Olubukola³, Oluwadun Afolabi³, Antonio M.⁴ and Adeqbola. R. A.⁴

¹Biochemistry and Nutrition Division, Nigerian Institute of Medical Research, P. M. B. 2013, Yaba-Lagos, Nigeria. ²Molecular Biology and Biotechnology Division, Nigerian Institute of Medical Research, P. M. B. 2013, Yaba-Lagos, Nigeria.

³Olabisi Onabanjo University, Sagamu, Ogun State, Nigeria. ⁴Medical Research Council (MRC). Atlantic Boulevard, Fajara, Gambia.

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Nigeria is currently one of the highest pneumococcal disease burdened countries not implementing routine pneumococcal conjugate vaccine (PCV) immunization and having limited clino-biological data on Streptococcus pneumoniae. This retrospective study provides phenotypic and genotypic data on 15 isolates of S. pneumoniae recovered from clinical samples provided by 75 bacteremia, asthma, pneumonia, otitis media, meningitis, severe malaria and sickle cell anaemia (SCA) patients, attending health facilities within the south-West region of the country. The recovered S. pneumoniae isolates were serotyped and had their antibiotic susceptibilities determined by disk diffusion and MIC assays. They were further analyzed for disparity by SDS-PAGE and RAPD analysis coupled with genotyping for ply and lyt genes to query virulence. Empirical antibiotic prescription and demographic data were also extracted from the patients' medical records with consent. The 15 recovered S pneumoniae isolates belonged to 5 distinct serotypes: 19F (n = 6), 5 (n = 3) and 2 each of 6B, 9V and 23F. More isolates were recovered from children than adults and from invasive diseases than non-invasive ones. However, serotype 9V isolates (adults only) were distinctively invasive and genotyping revealed some levels of clonal diversity and virulence among the multi-drug resistant strains. All the strains were within the vaccine coverage of PCV-13.

Key words: Streptococcus pneumoniae, serotypes, antibiotic susceptibility, random amplified polymorphic DNA (RAPD), pneumococcal conjugate vaccine coverage, Nigeria.

INTRODUCTION

Streptococcus pneumoniae remains a leading cause of morbidity and mortality from meningitis, bacteremia and pneumonia in Nigerian children, aged-adults and the immuno-compromised such as sickle cell anemia and HIV patients (Akuse, 1996; Adeleye et al., 2008; Obaro, 2009). Currently, pneumonia, which is one of the diseases caused by S. pneumoniae, kills 200,000 Nigerian children below 5 years annually (Onche, 2009). This makes Nigeria a high pneumonia burdened country

where children are 17 - 400 times more likely to die from pneumonia than a child living in the US 'World Pneumonia Day Media Report, 2010'. Recent studies in Nigeria have also implicated S. pneumoniae as the cause of 13% of overall deaths in north central Nigeria and 80% of deaths from meningitis in Ibadan, both occurring in children below 5 years (Falade et al., 2009; Obaro et al., 2011).

Like in other endemic countries of the world, where three-quarter of global pneumonia deaths occur, risk that have been identified for invasive factors pneumococcal diseases (IPD) due to S. pneumoniae in Nigeria include air pollution, overcrowding, nasopharyngeal carriage and high level transmission of

^{*}Corresponding author. E-mail: bamwal@yahoo.com. Tel: +234-1-8023357646.

the pathogen as well as the presence of co-morbidities such as HIV/AIDS and sickle cell anemia (Akuse, 1996; Akinsete et al., 1998; Media report, 2011; Adetifa et al., 2012). Deaths resulting from *S. pneumoniae* infections have been attributed to its capsular polysaccharide cell wall that gives rise to over 90 serotypes, and protein factors such as autolysin (*lytA*) and pneumolysin (*ply*) that are involved in invasion, disease progression, and protection from host mediated opsonization and phagocytic killing (Appelbaum, 1992). Furthermore, the country is also plagued by an inadequate health system with sub-optimal vaccine coverage that is presently at 72% for non-pneumococcal vaccines (WHO-UNICEF, 2010), disease surveillance and health system research in the last 20 years (Uneke et al., 2010).

Meanwhile in pneumonia burdened countries, two-third of annual deaths could be averted if 90% of children had access to simple and effective pneumococcal vaccine (Media Report. 2011). Therefore, to address the pneumococcal problem in response to the global call for action against pneumonia, Nigeria has planned to integrate pneumococcal conjugate vaccine (PCV) into her national immunization program (NIP) in 2013 and etiology promote research for improved case management and preventive measures. Expectations of benefits from PCV usage is based on the lessons learned from the presently implementing countries worldwide in which life-year, quality adjusted life year and reduction in colonization, transmission and burden of invasive pneumococcal diseases (IPDs) in the post PCV era have been reported (Falade et al., 2009; Adetifa et al., 2012).

In the meantime, serotype data of circulating S. pneumoniae strains in the country are needed for making PCV procurement decision, evaluating serotype-specific disease burden and establishing baseline indicators to monitor and evaluate for outcomes and impact of prevention and surveillance programmes in the post PCV era. Information on drug response and clonal diversity of the circulating strains are also needed for better understanding of S. pneumoniae epidemiology and development of control program and policy in Nigeria. It is in this context that the present study was conducted with the aim of having a snapshot on the gene diversity by randomly amplified polymorphic DNA (RAPD) and whole cell protein profiling of S. pneumoniae strains form South-West, Nigeria. Sensitivity to anti-pneumococcal antibiotics by these isolates was also evaluated.

METHODOLOGY

Study design

This was a retrospective study of 15 *S. pneumoniae* isolates recovered from clinical samples that were obtained from 75 patients aged 1 - 62 years at 4 health facilities in Lagos and Ibadan, Oyo State, between March to July, 2010 and June 2011. The health facilities attended by the patients in Lagos were Massey Street Children Hospital, Lagos University Teaching Hospital (LASUTH),

and Onikan Health Centre, Lagos, while the cultured clinical samples from Ibadan came from patients attending University College Hospital (UCH). The presenting clinical conditions of the patients were severe malaria, sickle cell crisis associated pneumonia, bacteremia, otitis media, asthma, meningitis and chronic sinusitis. Pneumonia was diagnosed on the basis of chest radiological findings; positive Cerebrospinal fluid (CSF) culture results defined some meningitis cases, while bacteremia was based on positive blood culture results. These case definitions of patients included the use of clinical symptoms and case definition guidelines of Pneumococcal Vaccines Accelerated Development Plan (PneumoADIP) (Falade et al., 2009). Informed consent was obtained from the patients (adults) or their guardians for children before sample collection.

This study is ancillary to the respiratory pathogen surveillance study (Akinloye et al., 2011) approved by the Ethical Committee of the Oyo State Ministry of Health. The clinical samples submitted by the patients from whom *S. pneumoniae* strains were isolated were blood, cerebrospinal fluid (CSF), sputa (Bartlett grading complaint) and middle ear exudates according to their presenting clinical conditions.

Bacteriology and serological testing

Samples were cultured on 5% sheep blood agar (SBA) and chocolate agar plates by direct inoculation using a sterile loop. Blood samples (1 ml per patient) were cultured in trypticase soy broth (TSB) and Brain Heart Infusion broth (BHI) for 24 to 48 h for 7 days with sub-culturing on the agar plates on days 2, 3 and 7. The inoculated plates were incubated aerobically (5% sheep blood agar plates) and in a 5% CO₂ candle jar (chocolate agar plate) at 37°C for 24 to 28 h. The plates were examined thereafter for bacterial growth and positive plates were submitted to morphological evaluation by Gram staining and S. pneumoniae biochemical tests such as optochin sensitivity (IZD > 14 mm), 2% deoxycholate solubility and alpha haemolysis. To further confirmed the recovered S. pneumoniae isolates, API 20 Strep system (bioMerieux, France) according to manufacturer's directive was used. Serotyping was done using capsular and human factor sera based on antibodycoated latex agglutination assay (Denka, Seiken, Japan).

Antibiotic susceptibility testing (AST)

The isolates were screened for sensitivity to antibiotics by disc diffusion method on Mueller-Hinton agar supplemented with 5% defibrinated horse blood and nicotinamide adenine dinucleotide (20 ug/ml, Sigma, USA) using overnight culture from pure colonies of S. pneumoniae (on sheep blood agar plate) suspended in Mueller Hinton (MH) broth at 0.5 MacFarland standard density equivalent and standard antibiotic disks from Oxoid, UK, as follows: oxacillin, 1 μg; erythromycin, 15 μg; amoxicillin-clavulanate (AMC, 20/10 μg); tetracycline, 30 ug; trimethoprim/sulphamethoxazole (COT, 1.25/23.75 µg); chloramphenicol, 30 µg; cefotaxime, 30 µg; ceftriaxone, 30 µg; ciprofloxacin, 5 µg and laevofloxacin, 5 µg. After disk mounting, the inoculated plates were incubated at 35°C for 20 h under 5% CO₂ atmosphere. Plates were then examined for zones of inhibition with diameter produced measured in millimeters (mm) and interpreted as sensitive, intermediate resistance and resistance according to antibiotic breakpoint inhibitory zone diameter interpretation guidelines of the Clinical Laboratory Standard Institute (CLSI, 2006). Isolates with oxacillin zone sizes of >20 mm and < 19 mm were interpreted as penicillin sensitive and resistant respectively, while those with COT zone sizes of > 19 mm, 16 -18 mm and \leq 15 mm were referred to as sensitive, intermediate resistant and resistant isolates. Erythromycin zone sizes of > 23 mm, 14 - 22 mm and < 13 mm were indicative of sensitive,

intermediate and resistant isolates, while chloramphenicol or tetracycline zone sizes of > 21 mm and < 20 mm were indicative of sensitive and resistant isolates.

Minimum inhibitory concentrations (MICs) for penicillin, amoxicillin-clavulanate, ceftriaxone and ciprofloxacin were determined by microbroth dilution method with results interpreted using antibiotic MIC breakpoints recommended by National Committee for Clinical Laboratory Standards (NCCLS, 1990) for S. pneumoniae. Briefly, penicillin MICs of \leq 0.06, 0.12 to 1 and \geq 2 µg/ml were indicative of sensitivity, intermediate resistance and resistance respectively, while for amoxicillin/clavulanate, MICs of < 2, > 4 and > 8 µg/ml defined sensitive, intermediate resistance and resistance respectively. Ceftriaxone MICs of < 1 and ≥ 2 µg/ml defined sensitive and resistance isolates, while for ciprofloxacin, MICs of < 1 and > 4 µg/ml were definitions for sensitive and resistant isolates, respectively. In both disk diffusion and MIC determination assays, S. pneumoniae ATCC 49619 with penicillin resistance phenotype was used for quality control.

DNA preparation

A loopful of *S. pneumoniae* colony on SBA was suspended in 200 μ L of 100 mM Tris-HCl buffer (pH 7.4), followed by the addition of proteinase k solution (0.5 mg/ml final concentration). The suspension was incubated at 37°C for 15 min, then boiled for 10 min and centrifuged at 8,000 rpm for 10 min after cooling. The resulting supernatant was then transferred to a fresh Eppendorf tube and 2.5 μ L was used as the DNA template for polymerase chain reaction (PCR) and RAPD PCR assays.

Virulence gene detection

Two S. *pneumoniae* virulence genes: *lytA* and *ply* were amplified as 308 and 329 bp products by PCR using gene specific primers as described by Nagai et al. (2001) and Salo et al. (1995). The primer pair for *lytA* amplification was 5'-CAA CCG TAC AGA ATG AAG CGG-3'- F and 5'-TTA TTC GTG CAA TAC TCG TGC G-3'-R, while that of ply was 5'-ATT TCT GTA ACA GCT ACC AAC GA-3'-F and 5'-GAA TTC CCT GTC TTT TCA AAG TC-3'-R. The PCR cycling conditions for the amplification of both genes were as follows: 94°C for 2 min, followed by 25 cycles of 94°C for 10 s, 58°C for 15 s and 72°C for 60 s and a final extension step at 72°C for 5 min. The PCR products were recovered by electrophoresis on ethidium bromide (0.5 µg/ml) pre-stained 2% agarose gel.

RAPD PCR

An arbitrary primer 1254 ((5'-CCGCAGCCAA-3) from Biomers (Germany) was used for RAPD-PCR assay of *S. pneumoniae* DNA sample (100 ng) using a modified protocol of Duarte et al. (2005) in a 25- μ L PCR reaction volume comprising dNTPs (200 μ M each), primer (20 picomole), MgCl₂ (3.0 mM) and Taq polymerase (2.5 U) in 1X PCR buffer (20 mM Tris-HCl (pH 8.3) + 50 mM KCl). The PCR conditions were as follows: 2 cycles of 94°C for 5 min, followed by 40 cycles of 94°C for 1 min, 37°C for 1 min and 72°C for 2 min with a final extension step at 72°C for 10 min. RAPD-PCR products were resolved by electrophoresis on ethidium bromide (0.5 μ g/ml) pre-stained 1.0% agarose gel using a 1 Kb DNA ladder (Fermentas) for size extrapolation. The RAPD profile of *S. pneumoniae* ATCC 49619 was used for quality control.

Analysis of cell protein profile by SDS-PAGE

Here bacterial cell pellet prepared by centrifuging (5,000 rpm for

10 min) *S. pneumoniae* BSA culture suspension in sterile water and decanting the supernatant was homogenized in 150 μ L sample buffer (10% glycerol, 2% SDS, 5% β -mercaptoethanol in Tris-HCl buffer (pH 6.8). The resulting homogenate was then boiled at 80°C for 10 min, followed by centrifugation at 8,000 rpm for 10 min. Electrophoresis of the resulting protein sample (15 μ L per well) was carried as described by Merquior et al. (1994) using 4 and 12.5% stacking and running gel, respectively. The protein profiles were compared by visual inspection.

Discriminatory power of RAPD-PCR technique

This was carried out using the discriminatory index described by Hunter and Caston (1988) as given by the equation: $D = 1 - [1/N(N-1)]\sum nj (nj - 1)$, where D is the numerical index of discrimination, N is the total number of strains and nj is the number of strains pertaining to the jth type.

Data entry and analysis

Data obtained were entered and analyzed using SPSS 11.0 Statistical software. They were reported as number and percentages, median and range and mean (SD). Comparisons were done using chi-square, Fischer exact test, Kruskal-Wallis and Student's t-tests for percentages, median and mean values. Pvalues below 0.05 were considered to be significant.

RESULTS

Of the 75 patients aged 1 - 62 years who provided clinical samples for S. pneumoniae screening, 40 (53.3%) were children with 22 (55%) of these children aged < 5 years. Other age groups accounted for 46.7% of the clinical samples cultured (P>0.05). Overall, the female patients were significantly (P<0.05) younger than the males, but this was not evident in the children age group (age 1 -12 years). Gender difference was also observed in the frequency of otitis media and bacteremia with more females than males and vice versa. However, gender disparity in the isolation of 15 S. pneumoniae strains was not significant (P>0.05) (Table 1). Results presented in Table 2 showed that the 15 S. pneumoniae strains isolated were recovered from cases of meningitis (n = 4), suppurative otitis media (n = 4), bacteremia (n = 2), sickle cell anemia associated pneumonia (n = 2), asthma (n =2) and severe malaria (n = 1). Positive cultures were actually obtained from blood (n = 6), sputum (n = 2), ear swab (n = 3) and CSF (n = 2) samples respectively. The observed 40% isolation rate of the S. pneumoniae isolates from blood was also significant (P<0.05) when compared to other clinical samples. Furthermore, of the 15 recovered isolates, 3 came from Ibadan health facility, belonging to serotypes 5 (n = 1) and 19F (n = 2) from a patient with severe malaria and two patients with otitis media (Table 2)

The results in Table 3 and Figures 1 and 2 provide the summary of serotype affiliation, clonal differentiation, antibiotic resistance profiles and virulence disposition of the 15 recovered *S. pneumoniae* strains. Isolates

Table 1. Demographic and clinicopathological characteristics of the patients.

Variable	Male	Female	Total	Р
N (%)	40 (53.3)	35 (46.7)	75 (100)	> 0.05 ^a
Age, years				
Range (Median)	1-62 (12.5)	4 -35 (12)	1- 62 (12)	> 0.05 ^b
Mean (SEM)	19.2 (2.9)	13.3 (1.4)	16.4 (1.7)	< 0.05 ^c
Age distribution, n(%)				
1-2	3 (4)	0 (0)	3 (4)	ND
3-5	10 (13.3)	9 (12)	19 (25.3)	> 0.05 ^a
6-12	8 (10.7)	10 (13.3)	18 (24)	> 0.05 ^a
13-19	3 (4)	8 (10.7)	11 (14.7)	< 0.05 ^d
20-40	9 (12)	7 (9.3)	16 (21.3)	> 0.05 ^a
41 and above	7 (9.3)	1 (1.3)	8 (10.7)	< 0.05 ^d
Age group classification				
Children	21 (28)	19 (25.3)	40 (53.3)	> 0.05 ^a
Adults	19 (25.3)	16 (21.4)	35 (46.7)	> 0.05 ^a
Clinical condition				
S. pneumoniae, n(%)	9 (12)	6 (8)	15 (20)	> 0.05 ^a
SCA-Pneumonia, n(%)	6 (8)	4 (5.3)	10 (13.3)	> 0.05 ^d
Meningitis, n(%)	8 (10.7)	5 (6.7)	13 (17.4)	> 0.05 ^d
Asthma, n (%)	8 (10.7)	11 (14.7)	19 (25.4)	> 0.05 ^a
Supp. Otitis media, n(%)	1 (1.3)	7 (9.3)	8 (10.7)	< 0.05 ^d
Bacteremia, n(%)	13 (17.4)	2 (2.6)	15 (20)	< 0.05 ^d
Severe malaria, n(%)	4 (5.3)	6 (8)	10 (13.3)	> 0.05 ^d

Data are reported as number or percentages, mean (SD) and median (range). ^aChi-square test; ^bKruskall-Wallis ; ^cStudent's t-test;; ^dFischer exact test. P-values < 0.05 are significant.

Table 2. Distribution of isolated S. pneumoniae strains by samples and disease conditions and health facilities.

Cases/Samples (N)	Blood	Sputum	Ear exudates	CSF	Total
Severe Malaria, SM (10)	23F	-	-	-	1
SCA- Pneumonia (10)	5 , 23F	-	-	-	2
Bacteremia, Bac (8)	9V, 9V	-	-	-	2
Asthma, AS (19)	-	19F, 19F	-	-	2
Otitis media, OM (15)	-	-	6B, 19F, 19F, 19F	-	4
Meningitis, M (13)	6B	0	0	5, 5 19F	4
Total (75)	6(40) ^a	2 (13.3)	4 (26.7)	3 (20)	15 (100)

Data are numbers with overall percentages in parenthesis. Bolded serotypes are from Ibadan, while un-bolded serotypes are from Lagos health facilities. ^aP<0.05 compared to isolation rate from other clinical samples (Fischer exact test).

belonging to serotype 19F accounted for 40% (6 of 15 serotypes) of all serotypes recovered, followed by serotype 5 (3 of 15, 20%) and 2 each of serotypes 6B, 9V, 19F and 23F (Table 3).

RAPD and SDS-PAGE analyses revealed six and three distinct genotypes (1 - 6) and proteotypes (1 - 3) respectively, with most of 19F and 23F isolates belonging

indistinguishably to RAPD types 1 and 2 and proteotypes A and B, respectively. Overall, these isolates were positive for *ply* and *lyt* genes, suggesting that they are clinically important. Disk diffusion assays revealed that all the isolates elicited resistance to penicillin and tetracycline, while resistance rates to other resisted antibiotics were chloramphenicol (66.7%), erythromycin

Isolate	Age (year)	Case	RAPD type	Serotype	Protein profile	PEN	CHL	TET	ERY	TMP/SMX	AMC	CRO	CIP	LAEV	СТХ	lyt	ply
SP_01	3	М	1	19F	А	R	R	R	S	S	S	S	S	S	S	+	+
SP_02	1	Μ	1	19F	А	R	S	R	S	S	S	S	S	S	S	+	+
SP_03	4	Μ	2	19F	А	R	R	R	S	S	S	S	S	S	S	+	+
SP_04	23	OM	3	5	А	R	R	R	S	R	S	S	S	S	S	+	+
SP_05	42	Bac	NT	9V	В	R	R	R	R	R	R	S	S	S	S	+	+
SP_06	7	AS	4	5	С	R	R	R	R	R	S	S	S	S	S	+	+
SP_07	9	SCA-P	4	6B	А	R	S	R	R	S	R	S	S	S	S	+	+
SP_08	14	OM	5	5	А	R	R	R	S	R	R	S	S	S	S	+	+
SP_09	12	OM	2	23F	А	R	S	R	R	S	S	S	S	S	S	+	+
SP_10	2	SM	1	23F	В	R	S	R	R	S	S	S	S	S	S	+	+
SP_11	5	AS	6	6B	А	R	R	R	R	S	S	R	S	S	S	+	+
SP_12	11	OM	1	19F	В	R	R	R	S	R	R	S	S	S	S	+	+
SP_13	62	Bac	NT	9V	А	R	R	R	R	R	R	S	S	S	S	+	+
SP_14	4	Μ	1	19F	С	R	S	R	S	S	S	S	S	S	S	+	+
SP_15	8	SCA-P	5	19F	В	R	R	R	S	S	S	S	S	S	S	+	+
					R (%)	100	66 7	100	46 7	40	33.3	67	0	0	0		

Table 3. Distribution of S. pneumoniae isolates by serotypes, RAPD types, virulence expression and antibiotic resistance.

R% = Antibiotic resistance rate; SP_N (*Streptococcus pneumoniae*_strain no.); NT = not typeable; PEN = penicillin; ERY = erythromycin; AMC = augmentin; CRO = ceftriaxone; CTX = cefotaxime; CIP = ciprofloxacin; LAEV = levofloxacin; R = resistance; S = sensitive; + = detected; *ply* = pneumolysin gene; *lyt* = autolysin gene; SCA-P = sickle cell anaemia –pneumonia; M = meningitis; AS = asthma; OM = Otitis media; SM = severe malaria;

(46.7%), trimethoprim/sulfamethoxazole (TMP/ SMX) (40%), augmentin (33.3%) and ceftriaxone (6.7%). However, these isolates were sensitive to cefotaxime, ciprofloxacin and laevofloxacin (Table 3). Apart from the 23F and two of the 19F S. pneumoniae serotypes, other isolates were found to be multi-drug resistant and resulted in theproduction of 10 distinct serotype-dependent antibiotic resistance patterns with MAR indices of 0.2 - 0.6 with serotype 9V isolates as the most antibioticresistant strains. The latter was further confirmed by the MIC assays, which also revealed serotypes 23F and 6B strains to be more sensitive to the antibiotics tested and resistance to penicillin as intermediate for most isolates apart from serotype 9V and one of the three serotype 5

S. pneumoniae isolates recovered. Pneumococcal conjugate vaccine coverage rate analysis revealed coverage rates of 80 and 100% for PCV-7 and PCV-13, respectively (Table 4).

Microbroth dilution assays further confirmed that all the isolates except serotype 9V and 1 of the 3 serotype 5 strains had intermediate resistance to penicillin. Strains belonging to serotypes 23F and 6B were also observed to produce the lowest MICs for ceftriaxone, augmentin and ciprofloxacin, suggesting higher sensitivity when compared to other serotypes, while serotype 9V strains were the least sensitive to all the efficacious antibiotics tested (Table 5). However, isolates of serotypes 9V (adults only) and 5 (adult and children) were distinctively invasive and non-invasive in

aetiology, while serotypes 6B and 19F were associated with SCA-associated pneumonia Table 6). rate to erythromycin and trimethoprim/ sulfamethoxazole for invasive isolates over their non-invasive counterparts(Table 6). A total of 7 (46.6%) S. pneumoniae isolates were recovered from children < 5 years and 72.3% from children ≤ 13 years. The disparity in isolation rates between these age groups and between ≤ 5 and 14 - 40 or 41 and above age categories was significant (P<0.05) (Table 7). Empirical prescription records from 41 of the 75 patients showed that most empirical prescriptions were monotherapies, highest for trimethoprim/sulfamethoxazole (26.8%) and lowest for ceftriaxone (9.8%). Other empirically prescribed antibiotics include penicillin (19.5%),



Figure 1. Agarose gel electrophoresis of distinct RAPD patterns elicited by the *S. pneumoniae* isolates.

augmentin (14.6%), ampicillin (17.1%) and ampicillin + gentamicin (2.4%) Further analysis revealed significant (P < 0.05) resistance (Table 8)

DISCUSSION

In this study, we recovered 15 isolates of S. pneumoniae of serotypes 5, 6B, 9V, 19F and 23F from 75 cultured samples as aetiologic agents of invasive (bacteremia, meningitis, SCA associated pneumonia) and noninvasive (otitis media, asthma) diseases with more isolates recovered from children than adults. These isolates showed resistance to 2 or more antibiotics, genetic diversity by RAPD analysis and exhibited PCV-7 and PCV-13 coverage rates of 80 and 100%, respectively. Our isolation rate of 20% (15 of 75 samples) was lower than 24.9 and 37% reported by Lagunju et al. (2008) and Ndip et al. (1995), but higher than 6.4% reported by Agwu et al. (2006) in Ekpoma, Nigeria, in 2006, 11% by Adeleye et al. (2008) in Lagos in 2008 and 7.6% by Obaro et al. (2011) in north-central, Nigeria in 2011. Apart from the sample size with ours being the lowest, the observed difference in S. pneumonia isolation rates may also be due to difference in study design.

Agwu et al. (2006) surveyed *S. pneumoniae* among *Mycobacterium tuberculosis* infected patients, Adeleye et al. (2008) conducted a cross-sectional study of *S. pneumoniae* in HIV/AIDS patients, Obaro et al. (2011), screened for *S. pneumoniae* only in children aged \leq 5 years, while Lagunju et al. (2008) worked on children with meningitis only.



Figure 2. Whole cell protein profile by SDS-PACE of *Streptococcus pneumoniae* isolates screened. Wells with different letters represent distinct whole cell protein profiles.

In the work of Ndip et al. (1995), the S. pneumoniae isolates were recovered from patients with otitis media and lower respiratory tract infections. In the year 2000, the national estimate of pneumococcal disease burden was reported to be 5% in children below 5 years, while 27% was reported for India (Chawla et al., 2010). Nevertheless, our results have revealed persistence of S. pneumoniae as etiological agents of bacteremia, sepsis, meningitis, otitis media and pneumonia in Nigeria. It also portends an increase in the trend of S. pneumoniae disease burden in the country with potential variations in prevalence/rates by geographical and disease settings. Furthermore, the observed higher isolation rate of S. pneumoniae in children than adults agrees with previous findings in Nigeria and other endemic countries of the world (Adetifa et al., 2012; Kim et al., 2010). This may be attributed to early colonization of S. pneumoniae in children, usually by week five (Antonio et al., 2008).

An important finding of this study is the recovery of serotype 9V S. pneumoniae isolates in Lagos that were not reported by previous authors. Falade et al. (2009) recovered serotypes 19F, 4 and 5 isolates from children with sepsis, meningitis and pneumonia in Ibadan, Nigeria, while Onyemelukwe and Greenwood (1982) reported serotypes 1, 2, 3 and 5 S. pneumoniae isolates as causes of invasive pneumococcal diseases (IPD) in Nigeria in 1982. An exception to this discrepancy was the recent report by Adetifa et al. (2012). The workers serotype recovered 9V S. pneumoniae as nasopharyngeal carriage isolates, accounting for ~2% of all isolates recovered. Meanwhile, serotype 9V S. pneumoniae isolates are commonly isolated IPD pathogens in patients from Europe and the USA (Tracey et al., 1999; Jenkins et al., 2008). This serotype has also

Serotype	n	[@] MAR index	Resistance pattern	^MDR phenotype (%)	Coverage rate, % PCV-7 PCV -13
		0.4	PEN CHL TET TMP/SMX		
5	3	0.5	PEN CHL TET ERY TMP/SMX	100	80, 100
		0.5	PEN CHL TET TMP/SMX AMC		
	-	0.4	PEN TET ERY AMC	400	
6B	2	0.5	PEN CHL TET ERY CRO	100	
9V	2	0.6	PEN CHL TET ERY TMP/SMX AMC (2)	100	
		0.0			
		0.2	PEN TET (2)		
19F	6	0.3	PEN CHL TET ERY (3)	66.7	
		0.5	PEN CHL TET TMP/SMX AMC		
23F	2	0.3	PEN TET ERY (2)	100	

Table 4. Theoretical pneumococcal conjugate vaccine coverage rate, levels and serotype specific antibiotic resistance patterns among the isolated *S. pneumoniae* strains.

Figures in parentheses are number of resistance patterns. ^{s@}MAR index is calculated as the ratio of number of antibiotics resisted to total number of antibiotics tested; ^MDR phenotype is expressed as the percentage of total number of isolates eliciting resistance to 3 or more classes of antibiotics; Coverage rate is defined as the number of *S. pneumoniae* serotype recovered as a percentage of constituent serotypes in PCV-7 (4, 6B, 9V, 14. 18C, 19F, 23F) and PCV-13 (PCV-7 + 1, 2, 3, 5, 6C, 19A). n = Number of strains per serotype; PEN = penicillin; ERY = erythromycin; AMC = augmentin; TET = tetracycline; CHL = chloramphenicol; TMP/SMX = trimethoprim/sulfamethoxazole.

Table 5. Minimum inhibitory concentrations of tested antibiotics against the serotypes Streptococcus pneumoniae.

	Mean (SEM) MIC, μg/ml									
Serotype	Penicillin			Ceftriaxo	Ceftriaxone		Augmentin		Ciprofloxacin	
	S	I	R	S	R	S	R	S	R	
5	-	0.24 (0.07)	2.1 (0.07)	0.4 (0.05)	-	0.19 (0.05)	-	0.05 (0.007)	-	
6B	-	0.18 (0.07)	-	0.16 (0.07)	-	0.09 (0.03)	-	0.02 (0.006)	-	
9V	-	-	3.0 (0.7)	0.38 (0.1)	3.84	0.38 (0.1)	-	0.17 (0.07)	-	
19F	-	0.21 (0.07)	-	0.18 (0.03)	-	0.19 (0.05)	-	0.08 (0.02)	-	
23F	-	0.54 (0.1)	-	0.078 (0.02)	-	0.039 (0.01)	-	0.04 (0.002)	-	

Table 6. Evaluation of antibiotic resistance between invasive and non-invasive serotypes of Streptococcus pneumoniae isolates recovered.

Antibiotic	Invasive isolates [n = 9:5 (3), 6B, 23F (2), 9V (2), 19F] n (%)	Non-invasive isolate [n = 6B, 19F (5)] n (%)	P-value
Tetracycline	9 (100)	6 (100)	ND
Chloramphenicol	6 (66.7)	4 (66.7)	> 0.05
Penicillin	9 (100)	6 (100)	ND
Erythromycin	6 (66.7)	1 (16.7)	< 0.05
Trimethoprim/Sulfamethoxazole	5 (55.6)	1 (16.7)	< 0.05
Ceftriaxone	1 (11.1)	0 (0)	> 0.05
Cefotaxime	0 (0)	0(0)	> 0.05
Augmentin	5 (55.6)	0 (0)	ND
Ciprofloxacin	0(0	0(0)	ND
Laevofloxacin	0 (0)	0 (0)	ND

Age group (year)	S. pneumoniae, n (%)^
1-2	2 (13.3)
3-5	5 (33.3)
6-13	4 (26.7)
14-19	1 (6.7)
20-40	1 (6.7)
41 and above	2 (13.3)
Total	15 (100)

Table 7. Distribution of S. pneumoniae isolates by age among the infected patients.

^P<0.05 (1 - 5 vs. 6 - 13 or 41 and above age groups) according to Fischer exact test.

Table 8. Empirical antibiotic prescription rate among the patients studied

Antibiotic	Prescription^ n (%)
Penicillin	8 (19.5)
Trimethoprim/sulfametoxazole	11 (26.8)
Augmentin	6 (14.6)
*Ceftriaxone	4 (9.8)
Ciprofloxacin	5 (12.2)
[@] Chloramphenicol	5 (12.2)
[@] Gentamicin	3 (7.3)
Ampicillin	7 (17.1)
Ampicillin + Chloramphenicol	3 (7.3)
TMP/SMX + Chloramphenicol	2 (4.9)
TMP/SMX + Gentamicin	2 (4.9)
Ampicillin + Gentamicin	1 (2.4)

^Empirical prescription data were extracted for 41 patients. [®]Noted as ear drop applications. Ceftriaxone was administered empirically as an injection.

become increasingly important in South America as aetiologic agent of meningitis, bacteremia and sepsis (Camargos et al., 2006). Apart from the serotype 9V strains found in Lagos, the recovery of four other distinct serotypes (5, 6B, 19F and 23F), which has also been reported in previous studies, is a reflection of high level of serotype diversity among *S. pneumoniae* in circulation in Nigeria. Other serotypes that have been reported but not found in this study include serotypes 3, 4, 6A, 11, 14, 15C and 18C.

Our isolates were further differentiated into 6 RAPD types and 3 proteotypes, suggesting that they are clonally diverse. However, the clustering of serotypes 19F and 23F isolates within the same RAPD type and the presence of non-typeable serotype 9V isolates demonstrates the limitations of RAPD for better epidemiological characterization of *S. pneumoniae* isolates in this environment. Therefore, for better phylogenetic grouping of *S. pneumoniae* and improved understanding of serotype switching, typing techniques such as multilocus sequence typing (MLST) are required. The use of antibiotics remains an important component of

therapeutic management of patients infected with S. pneumoniae. In this study, all our isolates were resistant to penicillin by disk diffusion assay but with serotype 9V and 1 of the 3 recovered serotype 5 isolates actually toelicit absolute resistance by MIC. Our finding agrees with the baseline fact that oxacillin disk is inadequate in distinguishing intermediate resistance from absolute resistance of S. pneumoniae to penicillin (Chwla et al., 2010). The observed 100% resistance rate, each for penicillin and tetracycline and 40% for trimethoprim/ sulfamethoxazole were higher than 36, 21 and 14% reported for these antibiotics by Fashae et al. (2002) during a S. pneumoniae outbreak in Ibadan in 2002, but were comparable with respect tetracycline resistance by invasive isolates reported by Falade et al. (2009) in 2009. These workers also reported 100% resistance rate to trimethoprim/sulfamethoxazole by these pathogens. On the contrary, all the invasive isolates recovered by Obaro et al. (2011) in North Central Nigeria in 2011 were sensitive to augmentin and ceftriaxone as reported previously by Fashae et al. (2002).

Meanwhile, in Jos, also within the north central Nigeria,

resistance rates of 34.2% to erythromycin, 29.7% to penicillin and 10.8% to ciprofloxacin were reported for nasopharyngeal isolates of S. pneumoniae by Kandakai-Olukemi and Dido (2009). Furthermore, Ndip et al. (1995) had previously reported absolute sensitivity to penicillin and erythromycin by S. pneumoniae isolates causing otitis media in Lagos in 1995. In a hospital-based study by Akanbi et al. (2004) in 2002 in Ilorin, Nigeria, the recovered S. pneumoniae isolates were resistant to all the tested antibiotics, including penicillin (83%), erythromycin (56.6%), ceftriaxone (28%), ciprofloxacin (20%) and ampicillin (73.8%). In this study, resistance rates of 66.7, 46.7, 33.3 and 6.7% to chloramphenicol, erythromycin, augmentin and ceftriaxone were elicited by our isolates with all being sensitive to ciprofloxacin, levofloxacin and cefotaxime. Apart from confirming that antibiotics vary in their efficacy against S. pneumoniae according to site of colonization or infection, geographical location within a country and serotype affiliation (the latter was not shown by previous studies), our findings have further demonstrated changing trend in antibiotic susceptibility among the circulating serotypes of S. pneumoniae and confirm the need for continuous monitoring of S. pneumoniae for antibiotic resistance coupled with restricted use of antibiotics in the country (Agwu et al., 2006; Adeleye et al., 2008; Falade et al., 2009).

The extensive use, misuse and abuse of antibiotics have been identified as persistent promoting factors of drug resistance in developing countries of world, where access to drugs are poorly controlled and the level of self-medication remains high (Arikpo et al., 2011). In southern Nigeria, emergence of chloramphenicol and ampicillin resistant pathogens, including S. pneumoniae, Klebsiella pneumoniae, M. pneumoniae, and Moraxella catarrhalis responsible for neonatal meningitis was first reported in 1994, preceding the period of high use of chloramphenicol and ampicillin as essential medicines for the management of infectious diseases in the country (Akpede et al., 1994). In fact, empirical prescription is a standard clinical practice in Nigeria with the use of and antibiotics such as chloramphenicol and TMP/SMX as first line drugs in children under the integrated management of childhood illness (IMCI) scheme (Obaro et al., 201). Penicillin, erythromycin and trimethoprim/ sulfamethoxazole are also three distinct classes of antibiotics that are widely used prophylactically to prevent diseases and pneumococcal other opportunistic infections (e.g. Pneumocystis carinii infection) in Nigerian patients with sickle cell anaemia and HIV/AIDS (Grange et al., 2003). Therefore, our present antibiogram result, regarding these antibiotics is not surprising. After all, Adeleve et al. (2008) reported 100% resistance rate to TMP/SMX by S. pneumoniae isolates from HIV/AIDS patients in Lagos. The presence of substantial drug pressure by TMP/SMX in Nigeria may also be connected to the high use of the drug sulfamethoxazolepyrimethamine for intermittent preventative treatment (IPT) of malaria in pregnant women (Agomo et al., 2009) and by self-mediation at home in malaria management (Iwalokun et al., 2011). All over the world, resistance rate to penicillin by *S. pneumoniae* has increased progressively since the first report in 1967 in Australia (Hansman and Bullen, 1967). Apart from HIV seropositivity correlating with penicillin and TMP/SMX resistance by *S. pneumoniae* (Adetleye et al., 2008; Jenkins et al., 2008), other factors that have been identified by previous workers include prior use of β -lactam antibiotics, 3 months of hospitalization and a revious history of pneumonia (Crewe-Brown et al., 1997).

The present retrospective study has limitations in that most of these factors were not extracted for risk factor analysis. However, our empirical antibiotic usage data revealed empirical prescriptions of TMP/SMX, penicillin, augmentin and ceftriaxone to which resistance was observed among our isolates. This finding also confirms the role of antibiotic usage in the emergence and spread of drug resistant pathogens in endemic populations. Nigeria is one of your populations in which antibiotic usage without prescription and empirical prescription of antibiotics are high (Obaro et al., 2011). Therefore our findings warn against empirical prescriptions of antibiotics, particularly drugs like penicillin, TMP/SMX, antibiotics, β-lactams tetracyclines other and chloramphenicol against S. pneumoniae infections in these settings. In a situation where empirical therapy is inevitable, the use of third generation cephalosporins and fluoroquinolones such as ciprofloxacin and laevofloxacin is recommended in these settings. The possibility of using combination of two classes of antibiotics empirically is also advocated. But evidence based research is recommended to justify this. With regards to penicillin use, previous studies in other environments still support the use of this antibiotic against intermediate resistance serotypes based on the fact that penicillin is a class of antibiotics whose activity is time-dependent and with efficacy dependent on the duration of effective MIC between dosing. Since penicillin allows daily multiple dosing and is affordable, pharmacokinetics and pharmacodynamic data beyond MIC are urgently needed for its continued usage in our settings. It is equally important to monitor S. pneumoniae for emergence of new serotypes and incidence of serotype switching in our setting to guide the deployment of effective control measures. In this study, we found serotype 9V isolates which were not reported as invasive and non-invasive pathogens by previous workers (Adeleye et al., 2008; Obaro et al., 2011; Falade et al., 2009; Adetifa et al., 2012; Antonio et al., 2008). This suggests serotype evolution. Clinically, serotype 9V S. pneumoniae isolates were also found to be the most drug resistant, confirming the importance of serotyping in S. pneumoniae epidemiology and suggesting changing epidemiology of invasive S. pneumoniae infection coupled with the need

for continuous monitoring of these serotypes and emergence of new ones in Nigeria. Generally, the serotypes of S. pneumoniae recovered in this study are among the common 20 serotypes that account for over 70% of pneumococcal diseases due to S. pneumoniae worldwide. However, based on serotype composition, we found theoretical coverage rate of 80 and 100%, respectively for PCV-7 and PCV-13, suggesting that Nigeria will benefit more from PCV-13 than PCV-7 if adopted for routine vaccination to control pneumonia in the country. Currently, the global coverage rate of PCV-7 is less than 70% as it does reduce the burden of infections caused by S pneumoniae serotypes such as serotype 9V found in this study and serotypes 1, 3 and 4 reported previously in the country. Although PCVs are vaccines for children aged \leq 6 years, children above 6 years but with underlying medical condition who have not had PCV coverage (Scott et al., 2011), the ability of this category of pneumococcal vaccines to elicit herd immunity is an indirect benefit for controlling pneumococcal diseases in adults, particularly those of extreme ages, who are equally susceptible as children to S. pneumoniae infections (Kim et al., 2010). This benefit is a possibility in Nigeria as for other endemic countries who have integrated PCV into their national immunization programmes (Antonio et al., 2008; Kim et al., 2010). One evidence-based explanation for this explanation is the recovery of serotypes covered by PCV-13 in adults with bacteremia, also seen in this study and other pneumococcal diseases reported previously by other workers from Nigeria and other countries of the world.

Based on our findings and despite the small sample size of our investigation, we report circulation of multiple serotypes and multi-drug resistant *S. pneumoniae* strains in south-West Nigeria and a potential public health benefit of PCV-13 if adopted for routine use and integrated into the national immunization programme of the country. The use of better typing platform such as MLTS is also recommended for better understanding of clonal diversity, dissemination and pathogenicity of *S. pneumoniae* at regional, national and country levels.

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Full Length Research Paper

A histological study on the distribution of dermal collagen and elastic fibres in different regions of the body

Naveen Kumar¹, Pramod Kumar^{2*}, Keerthana Prasad³ and B. Satheesha Nayak¹

¹Department of Anatomy, Melaka Manipal Medical College (Manipal Campus), Manipal University, Manipal, India.
 ²Department of Plastic Surgery, Kasturba Medical College, Manipal, Manipal University, Manipal, India.
 ³Department of Information Science, Manipal College of Information Science, Manipal University, Manipal, India.

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The aim of the study was to assess the unequal distributions of dermal collagen and elastic content in skin sections perpendicular to each other from different regions of the body. We collected 20 skin samples from 10 regions of a cadaver. From each region, 2 samples were collected in 2 directions which were perpendicular to each other. Histological slides were prepared and stained for collagen and elastic fibres'. Photomicrographs taken from special stained sections were analysed using simple and reliable software "tissue quant" method. Various ratios were calculated. The quantitative fraction in terms of percentage area occupied by collagen and elastic fibres were observed to be different in different regions as well as in different directions of sections taken. This unequal distribution of the dermal collagen and elastic fibres in 2 different directions of the sections will possibly have an effect on wound healing process which in turn may have an effect on varied scar appearance and behaviour based on the region and direction of wound.

Key words: Collagen, elastic, tissue quant, image analysis, scar.

INTRODUCTION

The skin performs many vital roles both as a barrier and a regulator between the outside world and the internal environment. The physical toughness of the skin prevents the ingress of harmful chemicals and invading organisms, such as bacteria and viruses. It also provides resistance to shocks for the more sensitive tissues underneath.

Biochemically, the papillary dermis is composed of type III collagen and reticular dermis composed of type I collagen. Elastic fibres appear in dermis much later than the collagen fibres. They undergo significant changes during life. Changes in aging is best studied in non exposed skin, elastoid degeneration is the result of chronic sun exposure. In very old persons, fragmentation and disintegration of some of the elastic fibres may be

observed (David, 2009).

Much importance is attached to the appearance of skin, especially in our modern society. Medical conditions affecting the skin can have marked effects, not only on our state of well being but also on the ways we interact with other people and on our suitability for certain occupations.

Appearance of scar after the wound healing is a natural process. It is still a challenging task for the aesthetic surgeons to minimise the scar appearance in the procedures of wound healing. This is because of the fact that, scar tissue is composed of same collagen as in normal skin but with differences in arrangement pattern and composition (Sherratt, 2010). Scars in the skin are less resistant to ultraviolet (UV) radiation and sweat gland, and hair follicles do not grow back in scars.

Despite of the existence of sophisticated treatment and availability of multi drug therapy, the consequences following the wound healing resulting in scar appearance

^{*}Corresponding author. E-mail: pkumar86@hotmail.com. Tel: +91 820 2922192, +91 9901700052.

and its behaviour is mysterious task for both general and plastic surgeons.

Langer's line is the line of choice in surgical approach which explains the orientation of collagen fibres beneath the skin. However, differential content of connective tissue fibres in different regions of the body as well as differences in the same area between 2 different directions may also be responsible for the alteration in the appearance and behaviour of scar and should be considered.

Aims and objectives

1. To evaluate quantitative fraction of collagen and elastic fibres in skin samples that are cut in perpendicular direction to one another.

2. To analyse the significance of difference in collagen and elastic fibres in terms of percentage area in different regions of the human body.

3. To measure and compare the various ratios of collagen and elastic fibres in the dermis of skin sections perpendicular to one another in different parts of the body.

MATERIALS AND METHODS

Sample collection

Skin samples (20) were collected from 10 different regions of a formalin embalmed cadaver. From each region, two elliptical (1 \times 0.5 cm) full thickness skin sections perpendicular to each other were collected. Sample 1 was taken horizontally in all the regions except at joints and was marked as "horizontal". Sample 2 was taken adjacent to sample 1 area and in perpendicular direction to it. It was marked as "vertical". However, in the region of joint, section taken parallel to the joint represents "horizontal" and perpendicular to it as "vertical".

Skin samples were collected from following regions: Scalp (about 3 cm superior to auricular attachment), forehead (in the midline at the middle of the forehead), lateral canthus (immediately lateral to lateral angle of the eye), neck (in the midline at the middle of the front of the neck), shoulder joint (immediately lateral to bony landmark of coracoid process), chest (in the midline, roughly at the middle of the body of the sternum), wrist (on the dorsal surface about 2 cm above the wrist joint), abdomen (in the midline about 2 cm below the umbilicus), groin (at the midpoint region of inguinal ligament), and thigh (on the middle of the medial surface of the thigh).

The aforementioned regions of interest are the areas which are either cosmetically important or important from the point of view of placing scar for achieving more aesthetic scar.

Tissue processing

All the skin samples were processed for histological study. The following staining methods were used:

Haematoxylin and Eosin (H&E) staining: for overall histological appearance of connective tissue fibres in the dermis of the skin.
 Combined Verhoeff stain-Van Gieson stain: to demonstrate

elastic fibres (by Verhoeff stain) and collagen fibres (by Van Gieson stain).

Verhoeff-Van Gieson stained slides are further analysed for tissue quant-image analysis to analyse the percentage area distribution of collagen and elastic fibres which is referred to as quantitative fraction.

Processing and staining procedures were followed according to standard techniques explained by John (2002). Processed tissues were embedded with paraffin wax followed by section cutting using rotary microtome. Sections from each skin sample were stained with routine H&E staining and with combined Verhoeff-Van Gieson stain for elastic and collagen fibres.

Slides were observed under light microscope to assess the general pattern of distribution of collagen and elastic fibres in the dermis before being subjected to image analysis.

Image analysis by tissue quant method

The sections stained by Verhoeff-Van Gieson method, were subjected to image analysis using the tissue-quant software. For the image analysis by tissue quant method, photomicrographs (20× magnifications) were obtained by inverted phase contrast microscope. Images from 3 different fields were taken from each slide and were analysed. The staining property of the collagen and elastic fibres are processed by image analysis using simple and reliable software "tissue quant". The tissue quant is an in-house developed software wherein the collagen and the elastic fibres are segmented out of the image by appropriately adjusting the colour settings. The areas covered by these tissues are then calculated by the software in terms of the number of pixels. All the images were acquired under the same magnification. Analysis from minimum of 3 fields of image from each stained sections were done. Mean percentage of collagen and elastic concentrations were calculated. From the mean values, various ratios of collagen (C1/C2) and elastic fibres (E1/E2) between 2 directions were calculated.

RESULTS

The dermal morphology was studied under light microscope using both H&E and special stained sections for overall pattern of connective tissue fibres. It was found to be normal throughout except for the elastoid degeneration in some regions.

The tissue quant analysis gives the data of quantitative fraction of dermal connective tissue components in terms of percentage area occupied by them based on the image analysis (Figures 1 and 2).

Data of quantitative fraction by dermal collagen and elastic fibers in two directions of skin samples obtained from different regions

Results depicted in the Table 1 and its graphic representation (Figure 3) indicates that, the percentage area occupied by collagen is much higher in groin (49%) region in its horizontal section and lowest in similar section of lateral canthus (16.8%). Similarly, the higher value was recorded for elastic fibres in horizontal section of wrist (22.5%) and lowest in scalp (0.5%) of same section.



Figure 1. (a) Appearance of collagen and elastic fibres in the dermis of horizontal section of lateral canthus (Verhoeff-Vangieson method 20×). (b and c) Segmentation of collagen and elastic fibres in horizontal section of lateral canthus, respectively by tissue quant image analysis.



Figure 2. (a) Appearance of collagen and elastic fibres dermis in vertical section of lateral canthus (Verhoeff-Vangieson method 20×). (b and c) Segmentation of collagen and elastic fibres of in vertical section of lateral canthus respectively by tissue quant image analysis.

Comparison of quantitative fraction values of collagen and elastic fibres between horizontal and vertical sections

In this study, the regions such as lateral canthus, chest, forehead, shoulder joint and wrist show that the percentage area occupied by collagen fibre was higher in vertical (C2) sections than in horizontal (C1) sections (H<V), whereas, the percentage occupied by elastic fibres was more in horizontal (E1) sections than vertical (E2) sections of all the regions studied except in scalp (Figure 4).

Ratio analysis

The C1/C2 ratio is calculated by dividing C2 value by C1. Similar calculation has been applied for E1/E2, C1/E1 and C2/E2 ratios.

Various ratio values in the Table 2 are summarised as follows. The ratio value is depicted in the bracket. The quantitative fraction of dermal connective tissue fibres of the horizontal section in both cases, that is, collagen and elastic fibres were kept constant as 1, so that the values denoted here is the proportionate value of its vertical counterpart. Hence, it is very clear that, the percentage area occupied by collagen was higher in the sections taken in vertical direction than horizontal direction in regions such as lateral canthus (1.50 times more than horizontal counterpart), chest (1.36), scalp (1.02), forehead (1.25), shoulder joint (1.35), wrist (1.08), and thigh (1.32), whereas, it was the reverse in the case of abdomen and groin (0.79) and neck (0.76). In these regions, collagen content is more in horizontal direction of the sections than its vertical counterpart.

Similarly, the percentage area occupied by elastic fibres was higher in vertical sections than horizontal observed in all regions studied except in scalp and groin regions. This shows that the quantitative fraction of collagen and elastic fibres in different regions as well as in different sections is not proportionately distributed. This imbalanced distribution of collagen and elastic fibres may play a significant role in the formation and the behaviour of scars during wound healing process

DISCUSSION

The major problem identified in the clinical setup during the process of wound healing is the variant appearance and behaviour of scars. The scars may be hypertrophic or stretched. Scar formation is the natural part of healing process which results from biologic process of wound repair. Hypertrophic scars are due to collagen content and stretching is dependent on elastic content. Appearance of the scar varies from region to region, for example, scar over the back, shoulder joint often stretches and develops hypertrophy, and it also depends



Figure 3. Graphic representation of mean quantitative fraction of collagen and elastic fibres in horizontal and vertical skin samples of various regions.



Figure 4. Graphic representation of quantitative fraction values of collagen and elastic fibres between horizontal and vertical sections.

on wounds of one direction to another. It is well evident in clinical setup as wound of horizontal direction on forehead results in an acceptable scar, while in the same region with vertical direction it becomes more obvious scar.

Hence, clinically, the behaviour of scar depends on the site and direction of the wound on the skin. It indicates that scar appearance and behaviour is also dependent on the pattern of quantity and arrangement of underlying anatomy of the dermis. Our study findings indicate that, the collagen and elastic fibre content differs in different direction, not only from region to region, but also in the same region.

The wounds that are under constant deforming contractile forces usually result in unaesthetic scars because of underlying anatomy (Kotler, 2009). The factor responsible for this is the pattern of distribution of elastic fibres in the dermis, which differs from one direction to another direction in a same region. Vitellaro-Zuccarello et al. (1994) reported that the fractional volume of collagen fibres is always higher in females than in males except for the 2nd and 3rd decades of life.

Skin site	Direction of section	Collagen (%)	Elastic (%)
Latoral capthus	Horizontal	16.83	9.19
Lateral Caritrus	Vertical	25.29	1.82
Chest	Horizontal	23.85	9.10
0001	Vertical	32.36	4.33
	l la via a stal	00.00	0.50
Scalp	Horizontal	28.99	0.50
	vertical	29.46	0.73
	Horizontal	49.05	3 92
Groin	Vertical	38.92	3 90
	ventical	30.32	0.00
	Horizontal	38.86	2.29
Abdomen	Vertical	30.75	1.75
Neek	Horizontal	44.00	2.05
NECK	Vertical	33.63	1.22
Forehead	Horizontal	27.64	20.62
ronolad	Vertical	34.62	16.91
Shoulder joint	Horizontal	31.55	5.54
, ,	Vertical	42.50	2.91
	Harizantal	00.60	00 E4
Wrist		22.03	22.04
	vertical	24.47	14.61
	Horizontal	24 93	12 92
Thigh	Vertical	24.33	11 38
	Voltical	00.02	11.00

 Table 1. Mean values of quantitative fraction of collagen and elastic fibres in horizontal and vertical skin samples of various regions.

Table 2. Ratio analysis derived from the results of tissue-quant image analysis.

Skit site	C1/C2 ratio	E1/E2 ratio	C1/E1 ratio	C2/E2 ratio
Lateral canthus	1.50	0.20	0.55	0.07
Chest	1.36	0.48	0.38	0.13
Scalp	1.02	1.45	0.02	0.02
Groin	0.79	1.00	0.08	0.10
Abdomen	0.79	0.76	0.06	0.06
Neck	0.76	0.59	0.05	0.04
Forehead	1.25	0.82	0.75	0.49
Shoulder joint	1.35	0.53	0.18	0.07
Wrist	1.08	0.65	1.00	0.60
Thigh	1.32	0.88	0.52	0.34

C1, Quantitative fraction of collagen in horizontal section; C2, quantitative fraction of collagen in vertical section; E1, quantitative fraction of elastic in horizontal section; E2, quantitative fraction of elastic in vertical section.

Collagen fibres density increases with age in both sexes up to 30 to 40 years. In reticular dermis of both sexes, there is an increment of elastic fibres density in the 1st decade of life, followed by a drop particularly marked in males. This clinical observation reveals that scar problem is more in children and young age group patients. This may have some relation with the aforementioned reported values of collagen and elastic fibres.

Clinical research about collagen and elastic content of abdominal skin after surgical weight loss showed undamaged elastic fibre content and moderate increase in epigastrium. Preoperative obesity had a negative correlation with hypogastric collagen content (Simone et al., 2010). This shows that skin stretching due to obesity (fat deposition under the skin) is opposed by elastic fibres. After losing the fat from subcutaneous tissue, the skin becomes loose. Hence, it may be indirectly inferred that the elastic tissue content of skin exerts the stretching force on the scar and thus scar behaviour and appearance is altered in proportion to the force which may vary depending upon the elastic tissue content and inherent property of elastic tissue.

In a study conducted by Gogly et al. (1997), diameters of elastic fibres increased regularly with age in the skin between each age group. The area fraction occupied by skin elastic fibres increased significantly within age group. Collagen diameter in mid-dermis also increased strongly with age group. But the area fraction occupied by the collagen bundles significantly decreased from the age of 50 to 75 years. Since the clinical observation shows that the scar in children and young age group does not settle well and majority of time unacceptable, the aforementioned findings of this study indicates a strong correlation between scar appearance and behaviour with collagen and elastic content of the dermis. Excessive laving down of collagen and stretching of scar is more in more elastic skin (e.g. in children and young subjects) (Berman et al., 2008).

Although, the significance of cleavage lines of the skin is well known in plastic surgery, it is considered that there is still uncertainty about their exact location in certain parts such as in mammary region (Zanon and Harp 1993). According to Wilhelmi et al. (1999), many surgeons prefer Langer's lines. However, Kraissl preferred lines oriented perpendicular to the action of the underlying muscles. Later, Borges described relaxed skin tension lines, which follow furrows formed when the skin is relaxed. They also opine that these are only guidelines as there are many factors contributing in the formation and behavior of scars, including wrinkle and contour lines. Borges's and Kraissl's lines (not Langer's) may be the best guides for elective incisions of the face and body, respectively.

Conclusion

This study suggests that the distribution of dermal collagen and elastic fibres varies not only in different regions, but also in two different orientations of the sections taken in the same region. Hence, it may provide an anatomical basis for explanation to earlier experience that the scar placed in a particular direction in a given region gives better aesthetic result.

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